MANUAL
of
AQUATIC VIRAL ECOLOGY

*MAVE* Chapter 17, 2010, 166–181


# Purification of viruses by centrifugation

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## Abstract

Ultracentrifugation provides a means to concentrate, analyze, and purify viruses in solution, and therefore represents an invaluable tool for aquatic virologists. This chapter reviews the theory of ultracentrifugation and presents the technical knowledge necessary for an investigator to adapt or develop methods to meet his or her needs. Detailed protocols for the purification of viruses from culture lysates and vial assemblages from natural water samples are provided.

Centrifugation can be used to concentrate, analyze, and purify biological particles ranging in size from whole cells to macromolecules. Ultracentrifuges have played a vital role in virology because they provide sufficient gravitational force (hundreds-of-thousands to over a million × *g*) to efficiently sediment even the smallest viruses. Depending on the rotor used, viruses can be pelleted from homogeneous water samples as large as 40 mL in less than 10 min and from volumes greater than a liter in less than an hour. Ultracentrifugation of viruses in density gradients is particularly useful, since it allows one to both concentrate and purify viruses in solution, thereby avoiding the problems of resuspending viruses from a pellet.

There are a number of excellent publications detailing the extensive development of ultracentrifugation technique (Anderson and Cline 1967; Brakke 1967; Rickwood 1984). The physico-chemical properties of relatively few marine viruses have been reported to date, but there is a vast amount of pertinent technical information available from previous studies of terrestrial animal and plant viruses. It is not our intent to attempt to replace these invaluable publications, but instead to provide an accessible compilation of the most pertinent information and relevant techniques for researchers working in aquatic virology. In this chapter, we provide a broad overview of ultracentrifugation theory, approaches, and techniques. We also provide details of a few specific methods that we expect to be most generally useful to aquatic virologists. These specific methods are still very flexible according to the equipment available, sample material, and required outcome, and should be tailored according to each application for optimal results. The technical information provided below will aid the investigator in modifying the methods as required to meet his or her needs.

## A primer on centrifugation and density gradient separation

*The physics of centrifugation*—Centrifugation provides a means for achieving two goals through one approach: particles can be both concentrated and purified under centrifugal forces. To understand how this is achieved, we must first consider the physics of a sinking particle. According to Stokes' Law, the sedimentation velocity, v (m $s^{-1}$), of a falling sphere is given by:

$$v = [d^2 (\rho_p - \rho_m)g]/18\mu \quad (1)$$

where d is the diameter of the particle, $\rho_p$ is the density of the particle (kg $m^{-3}$), $\rho_m$ is the density of the liquid medium (kg $m^{-3}$), μ is the viscosity (Pa s), and *g* is gravitational acceleration (m $s^{-2}$). Under ultracentrifugation conditions, centrifugal forces dwarf, and thereby replace, gravitational forces. Therefore, the velocity of a particle under ultracentrifugation becomes:

$$v = [d^2 (\rho_p - \rho_m) \omega^2 r] / 18\mu \quad (2)$$

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***Acknowledgments***

Publication costs for the Manual of Aquatic Viral Ecology were provided by the Gordon and Betty Moore Foundation. This document is based on work partially supported by the U.S. National Science Foundation (NSF) to the Scientific Committee for Oceanographic Research under Grant OCE-0608600. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF.

The writing of this chapter was supported in part by grants from NSERC to JEL and from NSF to GFS (OCE-04-42664 and OCE-0826650) and to the Center for Microbial Oceanography: Research and Education (EF-0424599). The authors acknowledge the efforts of the anonymous peer reviewers and their suggestions to improve the manuscript.

ISBN 978-0-9845591-0-7, DOI 10.4319/mave.2010.978-0-9845591-0-7.166

*Suggested citation format:* Lawrence, J. E., and G. F. Steward. 2010. Purification of viruses by centrifugation, p. 166–181. *In* S. W. Wilhelm, M. G. Weinbauer, and C. A. Suttle [eds.], Manual of Aquatic Viral Ecology. ASLO.

where ω is the angular velocity (radians $s^{-1}$), and $r$ is the distance from the particle to the axis of rotation. From this equation, we see that a particle in an ultracentrifuge is subject to two dominating forces: buoyancy ($\rho_p - \rho_m$) and sedimenting forces ($\omega^2 r$), and is influenced by the viscosity of the fluid and size of the particle. The balance of these forces governs the behavior, or sedimentation, of a particle in a centrifugal field.

While the density of a particle is intuitively important in determining its sedimentation velocity because of its influence on relative buoyancy, another convenient, but less intuitive expression is the sedimentation coefficient ($s$):

$$s = v/\omega^2 r \quad (3)$$

Modern expressions of sedimentation coefficient are in Svedbergs ($S$), which are equivalent to $10^{-13}$ s. Used together with density, these terms provide a convenient means for comparing the sedimentation characteristics of virus particles and other biological particles (Table 1), and determining the most efficacious type of centrifugal separation for virus purification, as explained below.

*Types of centrifugal separations*—Centrifugal separations can be separated into two basic types: differential pelleting and zonal separations. Differential pelleting is most useful for crude separations of raw material where purity and yield are not critical. The method involves sedimenting particles out of solution, and either retaining the pellet or supernatant depending on where the material of interest is located (Fig. 1A). As predicted by the equations above, larger particles will sediment prior to smaller ones, and more dense particles prior to less dense ones. In addition, asymmetrical particles will sediment more slowly than spherical ones of the same mass and density. The separations are not clean, however, since the centrifugal force required to pellet large particles from the top of a sample will also pellet small particles from the bottom. The greater the difference in sedimentation rate between the particles being separated, the cleaner the preparation will be. The most common practical application of differential pelleting is removing cell debris from viral lysates before concentrating and purifying viruses in the supernatant by other means.

Viruses can be harvested from a sample by pelleting in order to concentrate them, separate them from small contaminants, or exchange buffer, but some viruses may be damaged during pelleting or resuspension. Furthermore, virus particles may be difficult to disaggregate after pelleting, which can adversely affect some subsequent purification steps or analyses. The sedimentation rate of the viruses will increase and become heterogeneous as a result of aggregation and the number of viruses will be underestimated by infectivity assays or epifluorescence microscopy. If one intends to simply extract viral nucleic acids or proteins, then pelleting of viruses may be a practical harvesting method. If active, undamaged, well-dispersed viruses free of contaminants are required, then alternative density-gradient techniques should be considered.

There are two types of zonal separations, both of which rely on density gradients: rate zonal and isopycnic. Rate-zonal centrifugation separates particles based on differences in their sedimentation coefficients ($s$), which, from Eq. 1, we see is a function of both particle size and density. In practice, differences in size dominate the differences in $s$ among most biological particles, since the range of densities is not large and $s$ varies as the square of particle diameter. Isopycnic separations discriminate among particles based solely on differences in buoyant density. In both techniques, centrifugation is carried out in a density gradient, which, among other functions, prevents mixing of the sample thereby ensuring that separated particles remain separated.

For rate zonal separations, a sample is introduced to the top of a density gradient. When subjected to centrifugal force, the sample components migrate through the gradient according to their $s$. Particles migrate at different speeds, resulting in greater distance between particles having different $s$ over time (Fig. 1B). Because the particles do not come to rest at equilibrium in the gradient, care must be taken so that the particles of interest do not pellet. For effective separations, the initial sample volume should be small (the sample layer should be only a few millimeters thick), because the sample zone continues to widen over time as a result of diffusion. Therefore, while rate zonal gradients eliminate problems associated with pelleting during the purification, a suitable concentration step that does not result in pelleting or aggregation must be employed prior to using this technique. Many different types of density gradient media may be employed for rate zonal separations (*see* "Density Gradient Media"). Choosing the appropriate medium requires matching the properties of the medium to one's specific application. In general, it is beneficial to employ media preparations of high viscosity for rate zonal separations because viscous forces will magnify differences in settling velocity between similar particles. Centrifugation parameters (relative centrifugal force and run-time) must be determined empirically for new virus applications.

In isopycnic (or equilibrium buoyant density) separations, particles migrate through the density gradient until they reach the point at which their density is equal to that of the surrounding medium (Fig. 1C). Media used for this type of separation must therefore be able to form a solution that is at least as dense as the viruses that are to be purified. Samples may be top-loaded or bottom-loaded in preformed density gradients, or homogenously mixed with a self-forming gradient medium before centrifugation. As particles approach their equilibrium position in a gradient, the difference in density between the particle and the medium decreases and, consequently, so does the migration rate of the particle. Particles become increasingly focused over time until the focusing force is balanced by diffusion. Achieving equilibrium, at which point the particles are most focused, can require long centrifugation runs under high $g$-forces. This method eliminates pelleting and aggregation problems with virus applications, and produces highly

**Table 1.** Density, diameter, and sedimentation coefficient (*S*) for subcellular entities.

| Subcellular entity | Sedimentation coefficient (*S*) | Diameter (μm) | Density (g/cm³) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | CsCl | Sucrose | Percoll | OptiPrep | Metrizamide | Ficoll |
| Nucleus | $10^6$ to $10^{7‡}$ | 3–12‡ | | >1.32* | 1.08–1.12* | 1.2* | 1.23† | |
| Mitochondria | $10^4$ to $5 \times 10^{4‡}$ | 0.5–4‡ | | 1.13–1.19* | 1.07–1.11* | 1.14* | | 1.14* |
| Lysosomes | $4 \times 10^3$ to $2 \times 10^{4‡}$ | 0.5–0.8‡ | | 1.21* | 1.04–1.11* | 1.12* | 1.12* | |
| Peroxisomes | $4 \times 10^{3‡}$ | 0.5–0.8‡ | | 1.23* | 1.05–1.07* | 1.2* | 1.24–1.27* | |
| Viruses | 42 to >1000 | 0.02–0.4 | 1.18–1.51 | 1.15–1.29 | 1.06–1.08 | 1.14–1.22 | 1.13–1.31 | 1.07–1.14 |
| Nucleic acids (free) | 3.5 to 100 | n/a | 1.7–1.95 | 1.6–1.75§ | | | 1.18–1.79* | |
| Ribosomes | 80 | 0.025 | | 1.4 | | | | |

*Hinton and Mullock (1997)
†Schmidt (1973)
‡Luttmann et al. (2006)
§Griffith (1994)

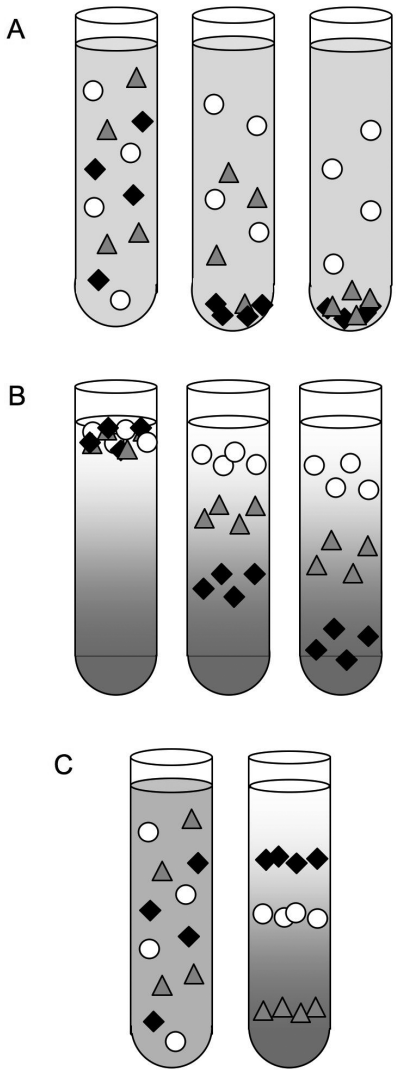


**Fig. 1.** Differential pelleting in a swinging-bucket rotor (A). Rate zonal centrifugation through a preformed gradient in a swinging-bucket rotor (B). Isopycnic separation through a self-generating gradient in a swinging-bucket rotor (C).

concentrated and purified virus preparations at the same time. Isopycnic separations also provide a means for directly determining buoyant density, a commonly reported physico-chemical property of viruses. One should be aware, however, that buoyant densities are specific only to the media in which they were determined.

Because the two zonal techniques described above separate based on partially independent properties (size versus density), they can be used sequentially to separate particles that may not be separable by either method alone (Fig. 2). Two-dimensional separations have been particularly valuable for virus purification, since most viruses have a combination of sedimentation coefficient and buoyant density that distinguishes them from most other cell constituents, including macromolecules and organelles. This region in the size-buoyant density space is referred to as the "viral window" (Anderson et al. 1966)

*Ultracentrifuge rotors*—Of the two major types of centrifuge rotors, conventional tube rotors have been used more commonly for aquatic virus purification than zonal rotors, perhaps because the former are cheaper to buy and simpler to use. In zonal rotors, the entire rotor bowl serves as the separation chamber. Vanes radiating from the axis in the chamber partially divide the bowl into compartments, which facilitates acceleration of the liquid mass while minimizing shear and mixing. With specialized fittings and a pump, fluid can be pumped in and out of the cylindrical cavity while the rotor is spinning (dynamic loading and unloading). Zonal rotors are technically challenging to operate, but have the advantage of permitting the processing of large volumes of sample as a single batch and permitting high-resolution separation of all components in a sample. Conventional tube rotors can handle volumes around 1/50 to 1/100 of a zonal rotor, but provide the highest yield of a single component in a mixture in the minimum length of time. With the wide range of tube sizes available, and the possibility of preconcentrating samples as needed, conventional tube rotors provide sufficient flexibility to accommodate most applications. This chapter will only address conventional tube rotor applications.

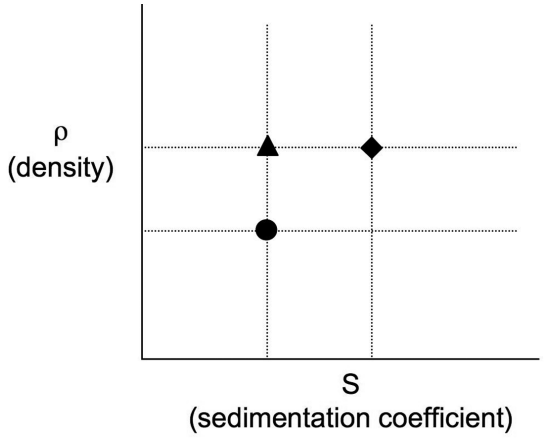


**Fig. 2.** Diagram showing the unique signatures of three viruses, V1(σ), V2(υ), and V3(λ). V1 and V2 are not separable on an isopycnic gradient, whereas V1 and V3 are not separable in a rate-zonal gradient. Since buoyant density and sedimentation coefficient are partially independent, rate zonal and isopycnic separations may be used sequentially to separate all three of these viruses from one another.

There are two main types of tube rotors: swinging-bucket and fixed-angle. In swinging-bucket rotors the sample and tube reorient to 90° from vertical during centrifugation such that the gradient is always parallel to the long axis of the tube. Fixed-angle rotors hold the tube in a fixed position and the liquid sample reorients within the tube during rotor acceleration and deceleration. Three sub-types of fixed-angle rotors are recognized based on the angle at which the tube is held. The term "fixed-angle" typically is used in reference to rotors in which the tube is oriented at an 18°-45° angle relative to vertical. Two other special cases of fixed-angle rotors are vertical (0°) and near-vertical (7.5°-9°) tube rotors. The differences in tube angle among these rotor types influences both the sedimentation path length (i.e., the maximum radial distance a particle must travel to reach the tube bottom or wall), and the range in magnitude of *g*-force within the tube.

A very useful descriptor of rotor performance that encapsulates particle path-length and centrifugal force is the *k* factor. Generally, the lower the *k* factor the shorter the runtime required to pellet the same particle. Table 2 provides the maximal *k* factors and maximal centrifugal forces for some commonly used rotors.

A very useful resource for modifications and adaptations of protocols is the Beckman Coulter Centrifugation Resource Center Web site. Applications include a rotor calculator, which permits conversions between *k* factor, centrifugal force, and rotations per minute (http://www.beckmancoulter.com/resourcecenter/labresources/centrifuges/rotorcalc.asp), and a runtime converter for different rotors (http://www.beckmancoulter.com/resourcecenter/labresources/centrifuges/runtimecon.asp). Alternatively, centrifugal force can be calculated using the following formula:

$$RCF_{max} = 1.12\, r_{max}\, (rpm/1000)^2 \quad (4)$$

where $RCF_{max}$ is the maximum rated speed of the rotor and $r_{max}$ is the maximal radial distance from the centrifugal axis. For calculating *k* factors when operating a rotor at less than maximal speed (in rpm), the following formula can be applied:

$$k_{adj} = k\ (\text{maximum rated speed of rotor / actual speed of rotor})^2 \quad (5)$$

Finally, runtimes can be converted between two rotors using the following formula:

$$k_1/t_1 = k_2/t_2 \quad (6)$$

The k factor can be used to calculate the time (*t* in hours) required to pellet all of the particles having or exceeding a given sedimentation coefficient (*s* in Svedberg units) when the rotor is run at its maximum speed:

$$t = k/s \quad (7)$$

This calculation, like that for *s*, assumes that sedimentation takes place in pure water at 20°C. Corrections can be applied to account for differences in the viscosity of water at other temperatures or for sedimentation in solutions other than pure water, and are best determined empirically.

All of the rotor types noted above can, in principle, be used for any of the separations described in the previous section, but best results are obtained when the rotor geometry is matched to the separation techniques being employed. Below we describe the main applications for each of the rotor types and some of their advantages and disadvantages for different types of separations.

- Swinging-bucket rotors. The path lengths in swinging bucket rotors are long and sedimenting particles have minimal interaction with the sides of the tube, making these rotors an excellent choice among tube rotors for rate-zonal separations. These rotors are also commonly used for isopycnic separations. In both cases, the length of the tube can be exploited to obtain high resolution among different types of particles. The large difference in *g*-force from the top to the bottom of the tube means that self-forming gradients are steeper in these rotors than in fixed angle rotors. This can be helpful when separating particles that differ greatly in buoyant density. Another advantage of swinging-bucker rotors for gradient applications is minimal disturbance to the gradient, since it does not reorient during the run. There is a rotational force on the gradient during acceleration and deceleration, however, so these are accomplished slowly. Swinging bucket rotors can also be used for pelleting particles. The potential advantage of pelleting in a swinging bucket rotor is that the pellet is compact and located at the very bottom of the tube. The disadvantage is that the time required to pellet material is much longer than in a fixed angle rotor.
- Fixed-angle rotors. The major application for fixed-angle rotors is pelleting. Since particles sediment to the wall, rather than the bottom of the tube, the path length is

**Table 2.** Specifications of swinging-bucket ultracentrifuge rotors commonly used for density gradients.

| Rotor | Maximum speed (rpm) | Maximum force (*g*) | *k* Factor (@ max *g*) | Rotor capacity (mL) |
|---|---|---|---|---|
| Beckman Coulter | | | | |
| MLS50 | 50,000 | 268,000 | 71 | 4 × 5 |
| SW28 | 28,000 | 141,000 | 245 | 6 × 38.5 |
| SW28.1 | 28,000 | 150,000 | 276 | 6 × 17 |
| SW32 Ti | 32,000 | 175,000 | 204 | 6 × 38.5 |
| SW32.1 Ti | 32,000 | 187,000 | 228 | 6 × 17 |
| SW40 Ti | 41,000 | 285,000 | 137 | 6 × 14 |
| SW41 Ti | 40,000 | 286,000 | 125 | 6 × 13.2 |
| SW55 Ti | 55,000 | 368,000 | 48 | 6 × 5 |
| SW60 | 60,000 | 485,000 | 45 | 6 × 4 |
| TLS55 | 55,000 | 259,000 | 50 | 4 × 2.2 |
| Sorvall | | | | |
| AH-629 (36 mL buckets) | 29,000 | 151,240 | 242 | 6 × 36 |
| AH-629 (17 mL buckets) | 29,000 | 155,850 | 284 | 6 × 17 |
| AH-650 | 50,000 | 296,010 | 53 | 6 × 5 |
| SureSpin 630/36 | 30,000 | 166,880 | 219 | 6 × 36 |
| SureSpin 630/17 | 30,000 | 166,880 | 268 | 6 × 17 |
| TH-641 | 41,000 | 287,660 | 114 | 6 × 13.2 |
| TH-660 | 60,000 | 488,580 | 44.4 | 6 × 4.4 |

shorter. The angle of the tube causes much of the material that reaches the wall to migrate down the wall to the most distal point within the tube near the bottom. Pellets are therefore relatively compact. Because of the wall effects, fixed angle rotors are not recommended for rate-zonal separations, but they can be used effectively for isopycnic separations.

- Vertical rotors. The major applications for vertical rotors are rapid isopycnic and rate-zonal separations. Because the gradient reorients by 90° within the tube during centrifugation, the path length for a tube in a vertical-rotor is the diameter rather than the length of the tube. The practical implication is that run times can be much shorter than in a swinging bucket rotor. The relatively small difference in *g*-force from $r_{min}$ to $r_{max}$, also means that self-forming gradients tend to be shallower than in swinging bucket rotors even at high speeds. A shallow gradient can be helpful when resolving particles that have small differences in buoyant density, but may be a disadvantage if one needs to separate particles with widely differing buoyant densities. The reorientation of the gradient in a vertical tube rotor has implications for the resolution. Generally speaking, the zones or bands in a swinging bucket rotor can be tighter than in a vertical rotor, but the space between bands of differing density can be greater in a vertical rotor. Vertical rotors are generally not used for pelleting, because the pelleted material is distributed along the entire length of the tube. This also means that any material that pellets during a gradient separation will be in contact with the entire gradient when the rotor comes to rest after the run.
- Near-vertical rotors. The very low angle of near-vertical rotors provides the benefits of a vertical rotor (short run times and minimal wall effects), while improving the purity of particles separated in a gradient. Contaminants that exceed the density range of the gradient during a zonal or isopycnic run will either pellet toward the most distal part of the tube near the bottom of the tube or float to the most axial part of the tube near the top, just as in a fixed-angle rotor. Material that is banded in the gradient near the center of the tube is then not in contact with these contaminants when the gradient reorients at the end of the run.

*Density gradient media*—Numerous investigators have identified the criteria for choosing density gradient media for biological separations (Cline and Ryel 1971; Hinton et al. 1974). In summary, the criteria are as follows:

- The media should be inert or at least nontoxic to the specimen (minimal osmotic effect, ionic strength, and neutral pH).
- The media should form a solution covering the density range for the particular application, and be stable in solution.
- The physical and chemical properties of the media should be known, and it be possible to use one or more properties to determine the precise concentration of the media.
- The solution should not interfere with monitoring of zones of fractionated material within the gradient.
- It should be easy to separate the sample from gradient material without loss of the sample or its activity.
- The gradient media should be available as a pure compound, and be relatively inexpensive.

Although viruses are not physiologically active, and therefore, less susceptible to some stresses than living cells, osmotic and ionic compatibilities are still important for maintaining the integrity of the virions, especially for membrane-bound viruses (Anderson et al. 1953). Here we summarize the properties and common applications of the most common types of density gradient media noting their advantages and disadvantages for specific applications. More detailed information on the properties of various gradient media can be found in a number of books and technical manuals (e.g., Rickwood 1984; Griffith 1994).

Salts of alkali metals (cesium, rubidium, lithium, etc. salts)—Ionic media. Alkali metal salts, such as cesium chloride, are most widely used for making isopycnic gradients with any standard technique including preformed or self-forming gradients. Metal salts can provide some of the densest preparations available, have a low viscosity, and their concentration in solution is easily measured by refractive index. The major drawbacks of alkali metals lie in their effects on biological activity; salt solutions have high ionic strengths, which disrupt protein-protein and nucleic acid-protein bonds, and have high osmolarities, affecting particle hydration. These factors inactivate many sensitive viruses. Therefore, metal salts should be limited to use with robust viruses or applications where high yield and purity are needed, but biological activity is not required. Salts can easily be removed by a variety of techniques, including dialysis and ultrafiltration. The range of reported densities of viruses in CsCl varies widely, from 1.15 to 1.55 g $mL^{-1}$ (Fauquet et al. 2005). Analyses of marine viral assemblages suggest that DNA-containing viruses in the ocean are dominated by those having densities in CsCl of between 1.39 and 1.46 g $mL^{-1}$ (Steward et al. 2000).

Small hydrophilic organic molecules (sucrose, glycerol, sorbitol, etc.)—Non-ionic media. Sucrose meets most of the criteria of an ideal medium for rate zonal separations, being biologically inert, stable, and relatively cheap. Due to its popularity as such, sucrose is very well characterized with respect to concentration, viscosity, density, and refractive index, making it easy to develop and adapt methods for uncharacterized viruses. While sucrose has little effect on intermolecular bonding and is non-ionic, high osmotic pressure may cause shrinkage in enveloped viruses and thereby affect infectivity in sensitive viruses. The high viscosity of sucrose at concentrations useful for virus separations may aid in separation between similarly sized particles under rate zonal conditions, but the high viscosity and relatively low density limits the application of sucrose and other small organic molecules in isopycnic separations of viruses. Other sugars, notably glycerol and sorbitol, have also been used effectively as rate zonal media. These gradients need to be preformed as solutions of small organic molecules do not generally form gradients when centrifuged.

High molecular-weight organics (Ficoll, dextran, glycogen, etc.). High molecular-weight polysaccharides do not penetrate intact biological membranes and have a lower osmolarity than solutions of monosaccharides. Therefore, these media may be especially useful when employed with sensitive, membrane-bound viruses. Unfortunately, due to the size of these polysaccharides, they cannot be removed from the sample by dialysis or ultrafiltration, so dilution and high-speed centrifugation are generally required, which are contraindicated with sensitive specimens as discussed above. Since polysaccharide media such as Ficoll (GE Healthcare) and dextran diffuse slowly, it is necessary to preform linear gradients using gradient mixers. This characteristic also ensures that gradients are quite stable once formed. The high viscosity of these media necessitates longer spin times than those of sucrose gradients.

Colloidal Silica (Percoll, Ludox, etc.). Colloidal silica suspensions such as Percoll (GE Healthcare) and Ludox (DuPont) are truly non-ionic media that can be used to rapidly generate self-forming gradients. These media are well characterized, permitting the use of refractive index for examining density profiles of gradients since absorption prohibits monitoring by UV light. Percoll density marker bead kits, available from a number of vendors (e.g., Sigma-Aldrich, product DMB-10), are useful for visually monitoring gradient profiles. Whereas colloidal media cannot be effectively filter sterilized, they may be autoclaved before being adjusted for osmolarity and can be used over a wide pH range (5.5-10 for Percoll). Percoll is commonly used for cell separations, because the suspension of colloidal silica can be prepared in almost any buffer required to maintain cell viability. Viruses can also be separated in gradients of colloidal silica (Pertoft et al. 1967), but removal of the gradient medium from the viruses following centrifugation is a challenge, since both the viruses and the medium are colloidal. Another limitation is that the silica particles may begin to pellet before smaller viruses have time to form discrete, purified bands. To remove Percoll from virus purifications requires dilution and high-speed differential centrifugation (i.e., 100,000 *g* for 2 h in a swinging bucket or 1.5 h in an angled rotor), which may lead to aggregation and deactivation of viruses, as previously discussed.

Iodinated organic compounds (Nycodenz, OptiPrep, and metrizamide). Iodinated compounds provide an excellent combination of biological inertness, a wide density range, and low viscosity, which allows for reduced spin times. These compounds, including Nycodenz (Axis-Shield), iodixanol (sold as OptiPrep by Axis-Shield), and metrizamide, are heat stable, autoclavable, and of minimal ionic strength. Working with OptiPrep is simplified as there is a near-linear relationship between concentration and osmolality. The iso-osmotic nature of OptiPrep helps to minimize morphological artifacts, which is useful if the ultrastructure of a virus is to be investigated by electron microscopy. Concentrations of Nycodenz above 30% are hyperosmotic, although less so than for sucrose. In OptiPrep, many viruses have a banding density of between 1.16 and 1.26 g $mL^{-1}$ (Vanden Berghe 1983). There are few analyses of the range of buoyant densities found in

natural marine viral communities using iodinated gradient media, but one analysis from coastal California seawater indicated that most of the viruses Nycodenz were in the range between 1.18 to 1.29, with a peak around 1.24 (Steward unpubl. data). Stable gradients of iodinated media will self-generate or can be created through the diffusion of step-gradients. The many advantages of iodinated gradient media, and OptiPrep in particular, make them an excellent choice for isopycnic separations of viruses. Extensive resources on the properties and applications of these gradient media are available from Axis-Shield, including application guides for their density gradient products, a reference database, and specific methodologies for OptiPrep density gradient purification of more than 30 viruses to date (http://www.axis-shield-density-gradient-media.com/).

*Gradient design*—The shape of a gradient, also known as design, refers to the density profile through the gradient tube. When selecting gradient design it is important to first identify the type of separation and density media to be employed. Once these have been established, the number options are greatly reduced.

There are two classifications for gradient design: discontinuous versus continuous, and preformed versus self-forming. Discontinuous gradients have discretely layered zones of isopycnic media and are preformed before the sample is introduced. These steps are created using underlayering or overlayering techniques, and may be distributed in many equal-volume, isopycnic layers throughout the gradient, referred to as a step gradient, or may involve only one or two steps, known as a density-barrier or cushion gradient. Step gradients are useful for isopycnic separations, and provide an excellent combined concentration and purification method when only one, uniform component in the sample is of interest; the preceding density steps filter unwanted particles of lesser density while the supporting density step collects the particles of interest, letting more dense particles pass through. Density-barrier gradients are also very effective for concentrating viruses from simple samples, as they provide a high-density cushion to concentrate viruses on without forming a hard pellet. The primary considerations when designing a step gradient is that the top step is dense enough to support the sample when it is loaded, and the sample volume should not exceed 2% to 3% of the entire gradient volume if the separations are rate zonal, because the sample zone increases during the run as a result of diffusion.

In continuous gradients, the density gradually increases down the tube, although the slope of the density may be linear, convex, or concave. The simplest shape is linear, where density increases at a constant rate with volume and resolving power decreases with the steepness of the slope. Linear gradients are least affected by diffusion, in terms of their shape, and may be generated with a simple apparatus or by diffusion of a step gradient (for details, *see* "Gradient Preparation"). Self-forming gradients, which can be prepared with media such as cesium chloride, Percoll, metrizamide, Nycodenz, or OptiPrep, tend to be nonlinear, and the gradient shape varies depending on the rotor type used. Self-generating gradients form if the sedimentation rate is faster than the diffusion rate. For self-forming, continuous gradients, Percoll provides the most time-efficient option; a Percoll gradient will form in 30 min at 10-20,000*g*, although the silica particles continue to pellet after this point, and therefore, the gradient is not very stable. Cesium chloride and iodinated compounds take hours to self-form at very high speeds (50-100,000*g*), but if run-time is an issue, these media can be used to make preformed, stable gradients. An additional type, the isokinetic gradient, is beyond the scope of this publication as it requires a priori knowledge of both s and ρ and is therefore generally not applicable to discovery-based research. For rate zonal separations, the best design is a continuous gradient to avoid artifactual sample bands created by discrete increases in density. Continuous gradients are also most useful for fractionating complex samples since the gradual increase in density allows for the greatest resolution of sample components. When designing continuous gradients for isopycnic separations, the maximum density should exceed all the particles in a sample, and the slope should be minimized to maximize resolution. Sample volume on isopycnic gradients is not as narrowly proscribed as for rate zonal separations, because the sample particles focus at their isopycnic points independent of sedimentation rate.

A few general points should be considered when developing and adapting density gradients. Media concentration does not always have a linear relationship with either density or viscosity, but sedimentation rate varies inversely with viscosity. Since viscous drag has an increasing effect on sedimentation rates as particles move down a gradient, the viscosity profile is more important than density profile for determining run-time when working with highly viscous media. Concentration profile is the least important factor for consideration. It is important to monitor gradients to ensure that they are optimized with respect to shape and are reproducible from run to run.

*Gradient preparation*—Preparing gradient solutions: Once one has determined the gradient medium and the gradient range, solutions of the appropriate densities must be prepared. Here we provide reference material to assist in the preparation of CsCl and iodixanol solutions. The densities of these solutions vary linearly as a function of concentration (Fig. 3). The concentration of the gradient medium required to achieve the desired solution density can be estimated from the graphs, but a more accurate concentration can be calculated from the linear regression functions for the curves. For CsCl the formula is:

$$\%\mathrm{CsCl} = (\rho_{\mathrm{solution}} - \rho_{\mathrm{solvent}}) \times 135 \qquad (8)$$

where %CsCl is the concentration of the CsCl solution (in g per 100 mL), $\rho_{\mathrm{solution}}$ is the desired density of the final solution (g mL$^{-1}$), and $\rho_{\mathrm{solvent}}$ is the density of the buffer in which the

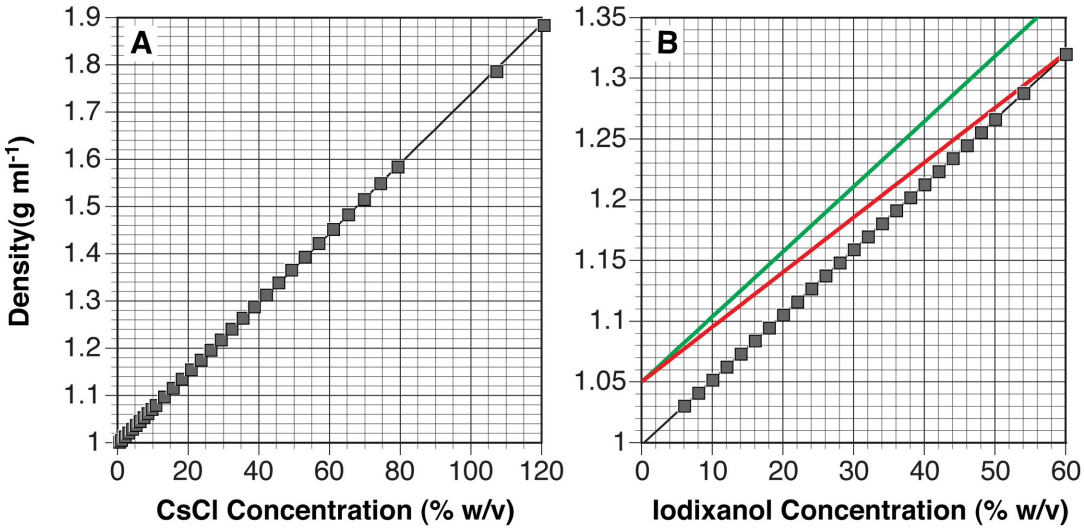


**Fig. 3.** Solution density at 20°C as function of the percent concentration of CsCl (A) or iodixanol (B) dissolved in water (solid squares). Black lines are regression lines for CsCl ($y = 0.007416x + 0.9982$) and iodixanol ($y = 0.005363x + 0.9982$). Note that the *y*-intercept represents the density of the diluent (water at 20°C has a density of 0.9982 g mL$^{-1}$). The linear regression formulae can be adapted for preparation and dilution of the gradient media in a buffer having a density greater than water by maintaining the slope, but changing the value of the intercept to reflect the density of the buffer. For example, dilution in a buffer with a density of 1.05 is illustrated (Panel B, green line; $y = 0.0054x + 1.05$). For OptiPrep™, which is supplied as a 60% solution of iodixanol in water, the function expressed by the green line assumes a stock solution in 1× buffer is first prepared as described in the text. If instead, the OptiPrep is simply diluted directly in 1× buffer, then both the slope and the intercept change, but the value at 60% remains fixed (Panel B, red line; $y = 0.0045x + 1.05$). Data for CsCl was obtained from Lide (2009) and that for iodixanol was derived from Axis-Shield PoC product literature (http://www.axis-shield-density-gradient-media.com/brochures.htm).

CsCl is being dissolved (g mL$^{-1}$). Since CsCl solutions are usually prepared from the solid, Eq. 7 can also be rearranged into a more convenient form for initial preparation of solutions of the desired density:

$$CsCl_{mass} = V_{solution} \times (\rho_{solution} - \rho_{solvent}) \times 1.35 \quad (9)$$

where $CsCl_{mass}$ is the mass of CsCl to be added (in g), and $V_{solution}$ is the final solution volume (in mL).

Iodixanol is supplied as a sterile 60% solution in water under the name OptiPrep and is usually diluted to ≤ 50% for most applications. To prepare a 54% stock solution in a 1× concentration of a desired buffer, combine 9 parts OptiPrep with 1 part 10× buffer. The concentration required to achieve a desired density can then be calculated as:

$$\%Iodixanol = (\rho_{solution} - \rho_{solvent}) \times 186.5 \quad (10)$$

where $\rho_{solvent}$ is the density of the 1× buffer being used for the dilutions.

Concentrated stocks of iodixanol (or CsCl) can be diluted to some desired lower density according to the formula:

$$V_{diluent} = V\ (\rho - \rho_{final})/(\rho_{final} - \rho_{\ diluent}) \quad (11)$$

where $V_{diluent}$ is the volume of diluent to be added, V and ρ are the volume and density of the solution to be diluted, $\rho_{final}$ is the desired final density, and $\rho_{\ diluent}$ is the density of the diluent.

The equations above call for the density of the diluent or buffer in which the gradient medium will be dissolved. One buffer commonly used for this purpose is SM (Sambrook and Russell 2001). Without the gelatin added, this buffer has density at 20°C of approximately 1.003. If one is preparing a gradient medium in some other buffer, particularly one with high concentrations of other solutes, the density can be determined empirically (*see* "Measuring fraction densities") or the equations can be modified accordingly as described in Fig. 3. It is good practice to verify the density of each solution once it has been prepared, because egregious errors can lead to loss of sample or, in the case of CsCl solutions, catastrophic rotor failure.

Gradient layering and sample loading. Making discontinuous gradients by hand is the most cost-effective option, and simple diffusion of many types of media creates continuous gradients from discontinuous ones. Underlayering each successively more dense solution results in the least amount of mixing between layers, providing the most discrete and reproducible gradient shape. Overlayering media layers is difficult to reproduce between tubes and runs, resulting in high variability between samples. For the best underlayering results, use a long, wide-bore pipetting needle (16-18 gauge, 4-inch cannula) on a disposable syringe. While resting the tip of the needle at the bottom of the tube, very slowly dispense the gradient media, being careful to not introduce any air bubbles as these will cause significant mixing (Fig. 4A).

For creating continuous gradients in open-topped tubes, step gradients can be covered and allowed to diffuse at room temperature for a couple of hours, in the refrigerator overnight, or even during centrifugation, provided the centrifugation time is sufficiently long. If the tube is sealed, diffusion in the

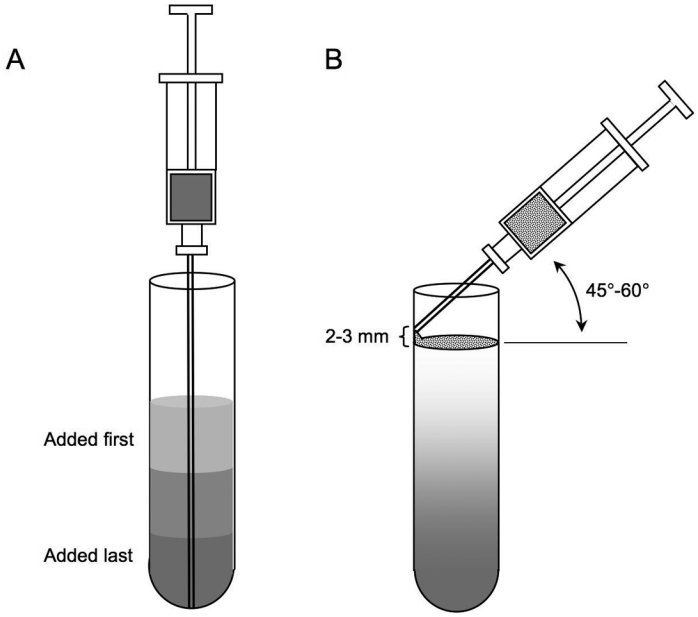


**Fig. 4.** Hand-layering a step gradient using the underlayering technique with a syringe and pipetting needle (A). Sample loading by overlayering onto a preformed gradient using a syringe (B).

horizontal position greatly speeds diffusion. Gradient mixers are also available from a number of suppliers, providing highly reproducible continuous gradients in much less time. Instruments include the Gradient Master and Gradient Station (Bio-Comp Instruments). The Auto Densi-FlowTM (Labconco) is an instrument that automates dispensing of gradients when connected to an appropriate gradient mixer.

For separations in self-forming gradients, samples are prepared as homogeneous solutions in the gradient medium. For separations in preformed gradients, samples are usually loaded onto gradients by overlayering. Samples should be overlayered using a narrow-tipped pipette or syringe with a narrow gauge needle. Keeping the delivery tip in the interface of the gradient, slowly dispense the sample to the surface, and centrifuge the sample immediately after loading to avoid diffusion (Fig. 4B).

*Gradient fractionation and sample collection*—Once a preparative gradient run has been completed, tubes should be handled gently, with as little rotation, tipping, or jarring as possible, and the sample recovered as quickly as practical. There are a number of methods for recovering samples from gradients. The method one chooses will depend on the nature of the separation, the type of centrifuge tube being used, and the equipment available. Most of these methods can be accomplished with little equipment, but the resolution and reproducibility may be improved with the use of specialized gradient-harvesting devices. Here we present methods for targeted harvesting of an individual band and for fraction collection.

Targeted harvesting of a single band.

- Side Puncture. If the desired band can be easily seen in the gradient, one may wish to selectively and directly harvest it. If the tube permits it, the tube may be punctured with a needle and syringe just below the band of interest with the needle hole pointed upward (Fig. 5A). Placing some clear adhesive tape over the desired puncture point before puncturing the tube helps maintain a seal around needle. The material is harvested by drawing liquid slowly into the syringe until the band is no longer visible. For sealed tubes, another puncture must be made near the top of the tube to relieve the vacuum during aspiration. A challenge with this method is making the puncture through the side of the tube with minimal disturbance. The tube will tend to deform from the pressure while forcing the needle through, and air bubbles can be introduced through puncture, which will rise through the gradient causing some mixing. If the needle is pushed directly through the wall of the tube, a plug of plastic may be cored from the wall of the tube and occlude the opening of the needle. To

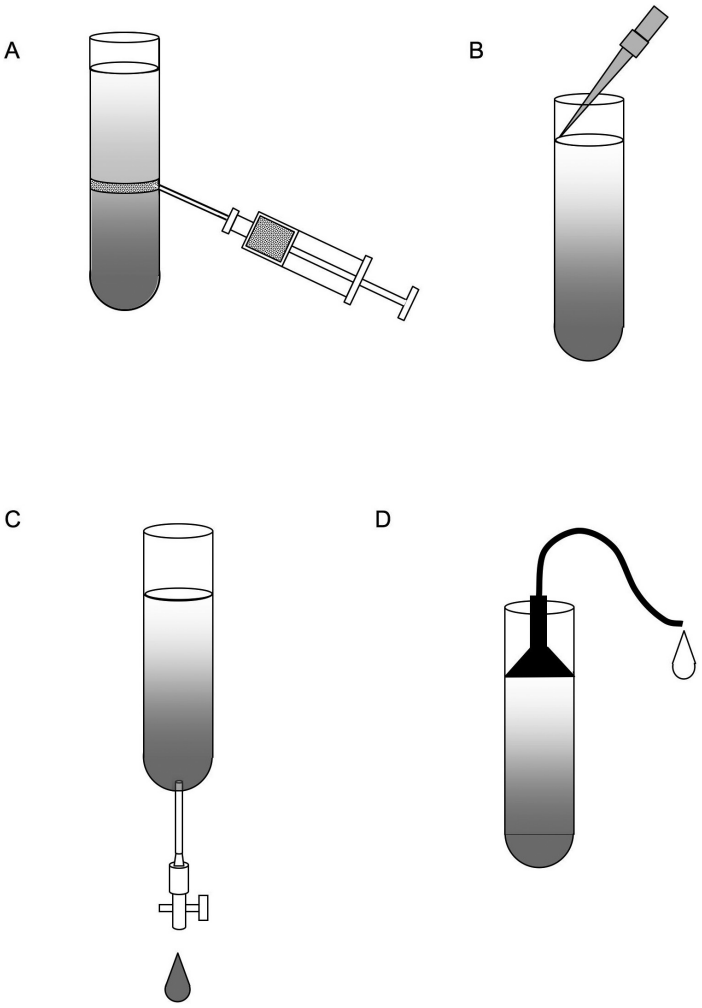


**Fig. 5.** Targeted harvesting of a single band in a density gradient by side puncture with a needle (A). Direct unloading of gradient fractions from the top with a pipette (B). Gravity drip collection with control through a bottom stopcock (C). Top unloading of fractions through a plunging, tapered piston (D).

avoid this, twist the needle as the tip penetrates the wall. The greatest hazard when using this technique is that the needle can slide quickly once it breaks through the wall of the tube. If one does not exercise control, the needle can penetrate the opposite side of the tube, and potentially one's finger if it happens to be in the path.

Gradient fractionating.

- Direct unloading. The simplest, but crudest method of collecting fractions from a gradient is to aspirate from the top of the gradient with a pipette. By keeping the pipette tip as close as possible to the meniscus during each aspiration, fractions can be collected with the least dense being removed first (Fig. 5B). This process can be automated using a device such as the Auto Densi-Flow, which has sipping tip with side holes that is attached to a motorized mount that moves the tip up and down. A sensor maintains the inlet holes of the sipper just below the meniscus, and a peristaltic pump connected to the tip drives the aspiration. A similar method is to insert a fine-gauge cannula or tube to the bottom of the gradient and draw the liquid out, starting with the most dense first. This is not recommended, however, since the gradient is inverted as it moves up the tubing, which leads to convective mixing and loss of resolution.
- Bottom puncture. A very effective method for harvesting fractions from a gradient that requires no special equipment is to simply puncture a hole in the bottom of the tube and harvest the drops from the bottom. It is most convenient to secure the tube above the bench (e.g., with a clamp on a ring stand) to free both hands for the tasks of tube piercing and fraction collection. The tube should be clamped with just enough force to keep it from slipping.

  Unloading a gradient through a bottom puncture can be accomplished several ways, the simplest being gravity drip. For gravity drip with sealed tubes, a hole will have to be made in the top of the tube to allow the pressure to equalize as the tube empties. This hole should be made before the bottom hole to relieve initial pressure differences between the inside and outside the tube. Pressure differences may result from squeezing of the tube in a clamp, or from a difference between the current temperature of the liquid in the tube and its temperature when the tube was sealed. If the bottom hole is made first, there may be an uncontrolled flow until the pressure is equalized. After the top hole is made, it can be sealed temporarily during the bottom puncture to prevent fluid from immediately flowing out. One can use a gloved finger to cover and uncover the upper hole to control the flow as fractions are collected by dripping into a series of tubes.

  A useful alternative means of flow control that also works for open top tubes is to pierce the bottom (or near the bottom) of the tube with a needle attached to a stopcock (Fig. 5C). Use a twisting motion during piercing to ease the needle through the wall and to avoid plugging the needle with plastic. A few drops may leak around the needle during the initial piercing, but these can be collected, if desired, by piercing at an angle and positioning a tube directly under the point of penetration. Adjust the position of the needle so that the opening is as near the bottom of the tube as possible. The stopcock can then be opened to give the desired flow rate.

  To provide an extra measure of control over the flow rate, the sample may be drawn from the bottom of the tube via peristaltic pump rather than relying on gravity. Alternatively, the gradient may be pushed out of the bottom piercing by displacement from above. In this case, the needle used to pierce the top of the tube remains in place and is connected to tubing passing through a peristaltic pump. Either air or pure water is then slowly pumped into the top of the tube to control flow through the bottom needle and stopcock assembly. Using this method with open top tubes would require that the tubes first be sealed with an appropriate size bung.
- Piston fractionator. An elegant solution for unloading gradients from open top tubes is to use a piston gradient fractionator (e.g., The Gradient Master). This device has a piston that seals against the walls of the tube. When the piston is pressed down, the liquid escapes through a hole in the tapered piston head and travels through tubing to the collection point (Fig. 5D). This programmable instrument allows reproducible harvesting of fractions as thin as 300 µm, and eliminates cross-contamination between fractions by rinsing and blowing-dry the sample tubing between sample points. The other advantage this instrument provides is by improving the visual detection of bands within a gradient. The sample holder supports the centrifuge tube in a bath of water, which renders the centrifuge tube transparent, and provides illumination from below. A Plexiglas window on the side of the sample holder allows the user to visualize and record banding patterns.
- Displacement from below. In this method, a liquid more dense than the densest part of the gradient is pumped into the bottom of the tube, which displaces liquid out the top of the tube through a specialized cap. Achieving this with a homemade set-up is not trivial, so this is typically accomplished with commercial gradient fractionators designed for the purpose (e.g., Beckman Coulter or Brandel). The dense solution can be added through a needle piercing the bottom of the tube, or by inserting a narrow tube to the bottom of the gradient from above. The latter method will cause some disturbance to the gradient during the insertion of the tube so is recommended only when collecting from thick-walled tubes that cannot be pierced.

*Measuring fraction densities*—It is often desirable to determine the density of a sample, whether to verify the density of stock solutions, determine the final shape of a gradient, or to estimate the buoyant densities of viruses harvested from gra-

dients. The relationship between density and concentration is well known for many common gradient materials and concentration can be determined quickly, accurately, and precisely using refractometry (Rickwood 1992). Disadvantages of this approach are that it requires specialized equipment and all samples contact the same surface of the instrument so there is the possibility for cross-contamination. This means that subsamples taken for measurement may have to be discarded, but the volumes required are not large. If the gradient media are prepared in a buffer, then one must correct for the refractive index of the buffer when calculating density. Refractometry is not recommended for measuring fraction densities if one is using mixed gradient media (e.g., sucrose and CsCl), because the relative concentrations of the two media will vary among the fractions after centrifugation, complicating interpretation of the refractive index.

Another simple, direct method for measuring density is to determine the mass of a known volume of sample using a precision balance and a micropipette with disposable tips. To make a mass measurement of a fraction, place the tube containing a fraction onto the balance and tare the balance. Then remove a fixed volume (e.g., 100 µL) with a pipette. Retain the volume that was removed in the pipette tip while the mass is recorded (as a negative number), then dispense the subsample back into the same tube. The advantages of this method are that no volume is lost, repeated measurements can be quickly made on the same fraction, and only the pipette tip must be changed between measurements of different fractions.

When determining density by direct mass measurements, one must bear in mind that air-displacement pipettes (the most common type) will systematically under-collect volume as density increases, leading to increasing underestimation of fraction densities. Since the error is systematic, it can be corrected for using an empirically determined function. The problem can be avoided by using a positive-displacement pipette. In a direct comparison of an air- versus a positive-displacement pipette, we found the latter was not only more accurate, but also more precise (Fig. 6). Whether the extra accuracy and precision obtainable with a positive-displacement pipette are worth the added cost of the tips will depend on one's application and the magnitude of the pipetting error relative to the many other sources of error (Rüst 1968).

Regardless of the method used to measure the density, the sample should be well mixed before taking a sample to make the measurement. Another important consideration when measuring density (or refractive index) is the temperature of the solution. When centrifuging cesium chloride solutions, one must pay careful attention to the combinations of CsCl concentration and centrifugation speed and temperature that allow safe operation. Charts are provided in the instruction manual for each rotor, but one must remember that the indicated maximum safe densities for a given speed and run temperature are the densities as measured at 20°C. If one plans to operate near the maximum allowable density, the measurements of initial density should be made close to 20°C or a correction factor should be applied to normalize the density to 20°C.

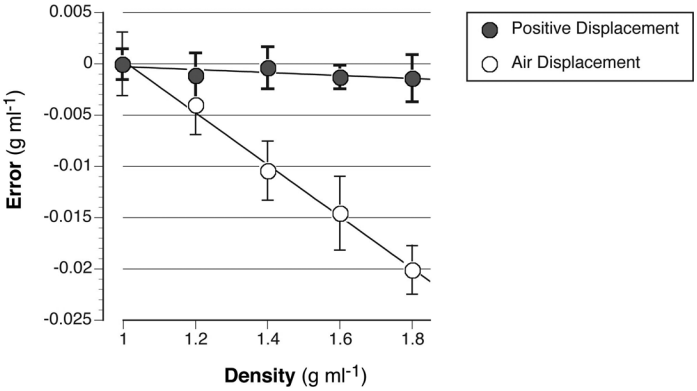


**Fig. 6.** Errors in calculated density as determined from mass measurements of 100 µL volumes using a positive-displacement or an air-displacement pipette. Error bars are standard deviations of triplicate measurements using three separate tips for each pipette. The increasing error as a function of density observed with the air-displacement pipette is attributed to an expected density-dependent undersampling. CsCl solutions of known density were prepared by combining measured masses of CsCl and water according to tables in the *Handbook of Chemistry and Physics* (Lide 2009).

If one wishes to estimate the buoyant density of particular viruses by measuring the density of the fractions in which they are found at peak concentration, one must account both for the temperature at which the density measurement is made, and the temperature at which the gradient was centrifuged, For example, if a virus is found in a fraction whose density was measured at 24°C, but the gradient was run at 4°C, one must account for the thermal expansion from 4°C to 24°C to determine the density at which the virus banded. The density of some viruses may also vary as function of pH or the time of exposure to CsCl (Rowlands et al. 1971), which could lead to variable estimates if centrifugation conditions change. One should also remember that the density of a fraction is an average for the entire fraction. The smaller the fractions, the more narrowly one can constrain the density of a peak. Finally, one must bear in mind that the diffusivity of CsCl is greater than that of viruses, so back-diffusion after the centrifuge has stopped has the potential to alter the apparent density profile and can lead to errors in estimates of virus buoyant density that are based on measurements of fraction density.

## *Applications in marine viral ecology*

*Purifying viruses from culture lysates*—In the typical culture lysate, viruses are a homogeneous and very abundant population of particles that are much smaller than the copious cellular debris, bacterial contaminants, and unlysed cells from which they need to be separated. While filtration is sometimes employed to clarify culture lysates prior to further purification, the amount of debris associated with lysed cultures often causes rapid changes in filter performance (i.e., nominal pore

size) resulting in the extensive loss of virus. Therefore, filtration is best avoided in favor of differential centrifugation. The specific conditions for clarification are quite variable, but 4000*g* for 30 min is a good starting point. If large-volume lysates are to be purified, it may then be necessary to concentrate the viruses prior to further purification. This can be achieved either with ultrafiltration techniques or through polyethylene glycol (PEG) precipitation. It is highly recommended that concentration only be undertaken after initial clarification of the lysate.

Once a relatively pure, concentrated sample of cultured virus has been obtained, final purification steps are best achieved through density gradients. A variety of approaches may be employed, each with relative strengths and weaknesses depending on the down-stream applications sought. However, if the virus of interest is not well characterized, it is well worth the effort to obtain infectious particles from the purification process so the isolated material can be confirmed as the infectious agent before further efforts are expended. The culture-based purification method described in Protocol A below aims toward this end.

*Purification of viruses from natural samples*—Purifying viruses directly from natural marine and freshwater samples is similar in many respects to purifying a virus from a culture lysate; the goal in both cases is to separate the viruses from as much of the contaminating material as possible. There are, however, a few additional considerations in working with natural samples. For many applications, one is interested in harvesting and purifying the entire viral assemblage, which is comprised of viruses having a broad range of physico-chemical properties. These diverse viruses must be separated from the heterogeneous collection of prokaryotic and eukaryotic cells that comprise the plankton. Depending on the purpose to which the purified viruses will be put, it may also be necessary to separate the viruses from organic and inorganic particulates and dissolved material. If the purpose of the purification is to perform molecular analyses of the viral nucleic acids, the requirement for purity may be more stringent, and viruses may need to be separated from both cells and from dissolved nucleic acids. Although viruses usually outnumber cells in the plankton by over an order of magnitude, their genomes are so small that their nucleic acids make up only a small percentage of cellular and dissolved pools.

The most common first step in purifying viruses from natural samples is filtration through a membrane having pore size of 0.2 or 0.22 µm. This removes virtually all of the cells, while allowing the vast majority of viruses to pass in the filtrate. The method is not perfect, as there are reports of ultramicrobacteria that can pass a 0.2 µm filter (reviewed by Velimirov 2001), and some marine viruses have diameters in excess of 0.2 µm (Bratbak et al. 1992; Garza and Suttle 1995). Viruses smaller than the pore size can also be trapped on the filter by adsorption or pore occlusion, resulting on occasion in low yields. Filtration is, however, very simple and effective for many purposes.

The alternatives to filtration are few, and each has its problems. One alternative directly relevant to the topic of this chapter is the separation of cells from viruses in buoyant density gradients. The great advantage of this approach is that, in principle, it allows the simultaneous separation of viruses from both cellular and dissolved DNA in one procedure, and there is no inherent selection against large viruses. In practice, the range of buoyant densities of viruses overlaps with that of cells, meaning that viruses having the lowest buoyant densities (those that are enveloped, contain lipids, or have a low mass percent of nucleic acid) may not be pure enough for some purposes. If minimizing cellular contamination is the priority, then filtration prior to gradient separation is recommended. If one is studying large viruses likely to be trapped on a 0.2 µm filter, but which have a buoyant density in CsCl that is > 1.35 g $mL^{-1}$, then direct loading of a whole-plankton concentrate onto a density gradient is an option worth exploring. Following up with other fractionation procedures may be required in that case to achieve the desired purity.

In the "Protocols" section, we describe a protocol ( "Purification of viral assemblages from seawater in CsCl gradients") for the purification of the viral assemblage from an aquatic samples, noting alternative procedures where appropriate. It is important to note that the exact conditions used can vary widely depending on your application. The basic principle of density gradient separation can easily accommodate some variations dictated by equipment availability. Effective separations can be achieved with either swinging-bucket or fixed-angle rotors, for example, although the time required for separation and the resolution of the gradient in the end will vary.

## Protocols

*Purification of viruses from culture lysates*—
Materials and reagents:

- OptiPrep (60% iodixanol solution)—Axis-Shield, Accurate Chemical and Scientific (Westbury), Progen Biotechnik, or Sigma Aldrich
- Open-topped ultracentrifuge tubes—i.e., Beckman Coulter Ultra-Clear
- Ultracentrifuge.
- Swing-out Ultracentrifuge Rotor—i.e., Beckman Coulter SW41, SW28, or MLS50
- 30 kDa cutoff disposable centrifugal ultrafiltration devices—i.e., Millipore
- 3-mL syringe with Luer-Lok or Luer-Slip
- Pipetting needle—i.e., Cadence Science, stainless-steel 14- or 16-guage 4-inch cannula with Luer hub or Slip hub
- Sterile 1.5 mL microcentrifuge tubes for collecting gradient fractions
- Sterile disposable transfer pipettes
- Sterile virus-free media for resuspending and diluting virus
- Polyethylene glycol, average molecular weight 6000-8000—i.e., Fisher Scientific Carbowax PEG 8000, or Sigma Aldrich Biochemika Ultra 8000

Steps:

Clarify lysate

1. Centrifuge the lysate at 4000*g* for 30 min.
2. Carefully decant and retain the supernatant.

Concentrate virus by PEG precipitation

3. Dissolve 8% PEG (w/v) in clarified lysate and allow to precipitate overnight at 4°C.
4. Centrifuge the PEG solution at 10,000*g* for 20 min.
5. Carefully decant the supernatant, retaining the pellet.
6. Resuspend pellet in a small volume of residual PEG solution and pool all pelleted material.
7. Repeat steps 5-6 as needed to concentrate virus to < 1 mL.
8. Resuspend virus in 10-50 volumes of culture media to dilute PEG and allow virus pellet to disaggregate overnight at 4°C.
9. Concentrate sample to ~1 mL through a 30 kDa cut-off disposable centrifugal ultrafiltration device.

Prepare continuous, isopycnic, purifying gradients

10. Prepare OptiPrep solutions using culture media as the diluent. For many viruses a gradient from 25%-40% OptiPrep will provide a good range for separation, but very dense or light viruses may require adjustment. To achieve this range, prepare 25%, 30%, 35%, and 40% v/v final-OptiPrep-concentration solutions, remembering that OptiPrep is sold as a 60% solution. The actual densities these concentrations achieve are dependent on the density of the culture media used as a diluent, and must be determined for each system.
11. Using the underlayering technique with syringe and pipetting needle, pour 4-step gradients into open-toped ultracentrifuge tubes, beginning with the least dense solution first. Be sure to leave enough room at the top of the centrifuge tube to load the sample, with 2-3 mm of space at the top. Allow to blend for 2 h at room temperature. Make sure to prepare gradients to serve as balance tubes where appropriate.
12. Mark the top of the gradients with a fine-tipped marker, and carefully overlay virus concentrate using a transfer pipette. Overlay culture media on balance gradients to create balance tubes.
13. Balance the tubes by adding media to underweight tubes.
14. Load tubes into rotor and ultracentrifuge at maximum permissible speed until density equilibrium is reached. As a guideline, a 4-mL gradient with 1-mL virus sample in a Beckman Coulter MLS-50 should be centrifuged for at least 4 h 15 min at 200,620*g* (50,000 rpm); an 11-mL gradient with 1-mL virus sample in a Beckman Coulter SW-41 should be centrifuged for at least 7 h 20 min at 207,570*g* (41,000 rpm). These conditions should be determined empirically for different systems.

Collect viral fraction

15. Using any fraction collection apparatus/technique, carefully extract purified viral concentrate from each tube. If bands are not visible, a starting point is to fractionate the gradient into 4 + fractions, and use a couple of techniques to identify the virus-containing fraction (i.e., TEM, bioassay, absorbance at 260 nm, nucleic acid analysis, epifluorescence microscopy or flow cytometry). Note that each of these techniques has drawbacks and may lead to false results. For example, more than one virus-containing fraction may be detected by TEM when analyzing non-axenic cultures, because contaminating bacteria are usually host to phage. Likewise, no virus-containing fractions may be detected by epifluorescence microscopy if the virus in question contains a small ssRNA genome, since the fluorescence yields of dyes are currently too low for visual detection of small ssRNA genomes. A combination of approaches for identifying virus fractions may therefore be required when working with novel viruses.

Discussion:

OptiPrep must be removed from samples before examination of virus particles by negative staining and TEM. This can be achieved using disposable Millipore centrifugal ultrafiltration devices with a 30 kDa cutoff. For most other applications OptiPrep does not need to be removed prior to further analysis, although it should be assayed to determine effects on the growth of specific viral-hosts when re-infection assays are used to confirm purification of the infectious agent.

*Purification of viral assemblages from seawater in CsCl gradients*—OptiPrep gradients, as described above for purifying viral isolates, may also be used for purifying marine viral assemblages. There are as yet, however, few descriptions of this application in the literature. CsCl gradients, on the other hand, have been used extensively for purifications of viral isolates and natural viral assemblages. Given the continued popularity of CsCl gradients, and their well-characterized performance, we present the following two-part purification and fractionation protocol for viruses in CsCl gradients. The first part involves relatively quick sedimentation through a step-gradient to remove the bulk of the contaminants. This is followed by a higher resolution continuous gradient to separate the viruses from residual contaminants and to separate viruses having differing buoyant densities from one another. We assume the starting material for the following procedure to be a concentrated suspension of viruses (whether filtered to remove cells or not).

Materials and Reagents:

- Ultracentrifuge
- Swinging-bucket rotor (Beckman Coulter SW 41 Ti or Sorvall TH-641)
- Polyallomer tubes (Beckman Coulter part No. 331372; Sorvall part No. 03669)
- CsCl (molecular biology grade)
- SM Buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris pH 7.5)

Steps:

Step gradient

1. Prepare CsCl solutions in SM buffer having densities of 1.2, 1.35, and 1.55 g $mL^{-1}$ (*see* "Gradient preparation" for helpful information)
2. Sequentially layer the CsCl solutions (2 mL of 1.55 g $mL^{-1}$; 2 mL of 1.35 g $mL^{-1}$; 1 mL of 1.2 g $mL^{-1}$) followed by the sample, into an ultracentrifuge tube. To minimize mixing at the interface where viruses will collect, the bottom layer (1.55 g $mL^{-1}$) can be chilled on ice prior to adding the next layer. The total volume should fill the tube to within 2-3 mm of the top. This procedure will accommodate up to 6.5 mL sample, but smaller sample sizes will ensure the most rapid sedimentation of viruses into the gradient. If necessary, top off the sample with mineral oil. NOTE: mineral oil is compatible with polyallomer tubes, but not Beckman Coulter Ultra-Clear or equivalent tubes.
3. Prepare a balance tube (containing a second sample or a blank) in the same manner as described above.
4. Verify that tubes that will be in opposing positions in the rotor are well matched. Opposing tubes should have the same layers that match in density and volume. The total masses of opposing tubes can be matched by adjusting the volume of the top layer. The final total masses should be matched to well within the tolerance of the rotor. Balancing to within 1% of the total mass should be more than adequate. Modern centrifuges have much greater imbalance tolerance (as high as 10%), but consult the manual for your rotor and centrifuge for recommendations and always err on the side of caution.
5. Centrifuge the sample at 40,000 rpm for 2.5 h at 20°C (4°C is also acceptable). Do this as soon as possible after the gradient has been prepared. For older rotors that have been permanently derated to 36,000 rpm, spin at this lower speed, and increase centrifugation time to 3.5 h.
6. Unload the gradient immediately after centrifugation by puncturing the side of the tube close to the bottom with a needle and stopcock assembly (*see* "Gradient Fractionation and Sample Collection") and collecting 0.5 mL fractions. A low side puncture is recommended, because some unencapsidated nucleic acids may have pelleted and could contaminate the virus fractions if the tube is punctured at the very bottom. Measure the densities of the collected fractions (*see* "Measuring fraction densities" for important considerations). Fractions having densities in the appropriate range can be pooled and subjected to further fractionation and purification. The majority of viruses in seawater have densities > 1.35 and < 1.50, while most bacteria will have densities < 1.35 g $mL^{-1}$. To ensure that the maximum numbers of viruses are recovered with the minimum contamination, one may wish to count viruses (and bacteria if appropriate) in each fraction by epifluorescence or electron microscopy. One could also assay the fractions for nucleic acid content to determine the distribution of RNA and DNA viruses, or by PCR to determine the location of specific viruses of particular interest.
7. If proceeding to the subsequent continuous gradient purification, pool the virus-containing fractions of the appropriate density and purity, make sure they are well mixed, then determine the initial density of the pooled sample (*see* "Measuring fraction densities") and proceed with the continuous gradient protocol below. If further purification is not required, proceed to the postgradient cleanup step described following the continuous gradient protocol.

Continuous gradient

1. Add CsCl and SM buffer as needed to the sample to achieve a final density of 1.45 g $mL^{-1}$ and a final volume of 4 mL. Make sure all of the CsCl is dissolved, the sample is well mixed and near 20°C, and then verify its density (*see* "Measuring fraction densities").
2. Prepare solutions of CsCl in SM having a density of 1.20 g $mL^{-1}$ (≥ 4.5 mL per sample) and 1.60 g $mL^{-1}$ (≥ 3 mL per sample).
3. Layer 3 mL of the 1.60 solution, followed by 4 mL sample at 1.45 g $mL^{-1}$, then 4.5 mL of the 1.20 g $mL^{-1}$ solution into an ultracentrifuge tube.
4. Prepare a balance tube (containing a second sample or a blank) in the same manner as described above.
5. Verify that tubes in opposing positions in the rotor are well matched. Opposing tubes should have the same density and volume and, therefore, total mass. The masses of opposing tubes should be matched to well within the tolerance of the rotor. Consult the manual for your rotor for recommendations, but within 1% of the total mass should be more than adequate (guidelines for modern centrifuges are 10%). Top off the gradients with CsCl (1.20 g $mL^{-1}$) as needed to achieve balance and to ensure that each tube is filled to within a few millimeters of the top.
6. Centrifuge the samples for ≥ 40 h at 30,000 rpm at 20°C (4°C is also acceptable).
7. Unload the gradient immediately, top end first, by direct unloading with an Auto DensiFlow, a piston fractionator, or by displacement from below (*see* "Gradient Fractionation and Sample Collection").
8. Screen the fractions for viruses (TEM, epifluorescence, nucleic acid assay, etc.) to determine which contain the viruses of interest. Alternatively, if the desired buoyant density range of the targeted viruses is known, the appropriate fractions can be selected

based on measured densities of the fractions (*see* "Measuring fraction densities" for important considerations when measuring fraction density).

The CsCl in the relevant virus-containing gradient fractions can be removed by buffer exchange using a centrifugal ultrafiltration unit with a nominal molecular weight cutoff of 30,000 to 100,000 (Microcon, Millipore or Nanosep, Pall Life Sciences). In this case, the sample is repeatedly concentrated to a small volume, then resuspended in the desired buffer. After several such buffer exchanges, the sample is resuspended and recovered in the final desired volume.

Discussion:

The point of the step gradient in this two-step protocol is to achieve a quick initial purification of viruses, which can then be purified to greater degree in the subsequent continuous gradient. The spin is kept short to minimize diffusion between layers, which would eventually result in a continuous gradient. Because the spin is short, not all material may reach its equilibrium position in the gradient. Viruses, having relatively high sedimentation coefficients, reach their equilibrium positions more quickly than many other dissolved macromolecules. Since all the material starts at the top of the gradient, viruses can therefore be most efficiently separated from less dense material (e.g., lipids, proteins, and most bacteria) as well as molecules that are more dense, but which have low sedimentation rates (small pieces of DNA or RNA). The subsequent continuous gradient, when centrifuged to equilibrium, provides good separation of viruses from contaminants and different viruses from one another. Since the less dense contaminants are mostly removed in the step gradient, which is unloaded from the bottom, we recommend unloading the subsequent continuous gradient from the top, thereby minimizing contamination from free nucleic acids, which will either band below the viruses (DNA) or form a pellet at the bottom of the tube (RNA). Viruses have been purified for metagenomic analyses using only a step gradient (Angly et al. 2006; Breitbart et al. 2004; Breitbart et al. 2002; Vega Thurber et al. 2009), or no gradient at all (Bench et al. 2007; Helton and Wommack 2009). In the latter cases 0.2-μm filtration and nuclease digestion are relied upon to remove nonviral nucleic acids. Since many virus genes recovered from the environment may not be recognized as viral based only on their sequence (Edwards and Rohwer 2005), having a highly purified virus preparation increases one's confidence that any novel sequences recovered do indeed derive from viruses rather than from cellular life forms. For that reason, one may find the complete two-part gradient purification protocol especially desirable for viral metagenomic studies. A rigorous gradient purification would be particularly important if the sample had not been first filtered to remove prokaryotes.

Fixed-angle rotors (including vertical, near-vertical, and others) are commonly used for equilibrium buoyant density gradients, especially self-forming gradients, because the centrifugation times required to approach the equilibrium gradient shape can be much shorter. However, the use of a swinging bucket rotor for the continuous gradient, as presented here, has several advantages: 1) only a single rotor is needed for the both the step and the continuous gradients, 2) the open-top tube simplifies gradient unloading from the top, and 3) there is less chance of contaminating the viruses with dissolved nucleic acids or cellular material. Any contaminating nucleic acid, especially RNA, that pellets during the run will be located at the very bottom of the tube where it will not be in contact with the virus bands. Material that is less dense than the least dense portion of the gradient will not pellet, but will float at the top of the gradient. In any type of fixed angle rotor, these potential contaminants will pellet to some degree on the sides of the tube and come in contact with the viral bands during deceleration as the gradient reorients in the tube. This material could contaminate the recovered fractions if it is dislodged or diffuses from the pellets during unloading. This is of particular concern for a sample that has not been first purified through a step gradient.

To partially compensate for the longer centrifugation times required for gradient to reach equilibrium in a swinging bucket rotor, we present a protocol in which a continuous gradient forms by diffusion from an initial step gradient. The modest amount of extra effort needed to prepare the step gradient is compensated by a significantly shorter run time. If one were to start with a homogeneous CsCl solution in an SW 41 or TH-1641 rotor, it could take ≥ 80 h for the gradient, and the constituents within it, to approach equilibrium. Another advantage of the step gradient is that it provides a dense cushion at the bottom of the tube that prevents viruses from pelleting early in the run before the gradient has fully formed.

WARNING! The protocol we present here is tailored to the specified rotors and operating conditions. Many other rotors and centrifugation conditions could be used instead. However, if you wish to change the conditions or adapt the methods to a different rotor, it is critical that you ensure that the new conditions are within the safe limits for centrifugation of CsCl gradients. Centrifugation of CsCl solutions at certain combinations of concentration, temperature, and rotor speed can result in CsCl crystallization at the bottom of the tube. The high density of the crystals will exceed the tolerance of the rotor and could result in catastrophic rotor failure.

*Summary*—Ultracentrifugation provides a highly flexible means for purifying viruses. While it is undoubtedly one of the most effective approaches, there are many critical variables to consider when developing and adapting methods for optimal performance. As with any technique, researchers who endeavor to apply ultracentrifugation should thoroughly consult the literature for previously developed methods intended for similar downstream applications, and be prepared to optimize for the particular material and equipment available. It cannot be emphasized enough how important it is to monitor each step in the purification process to ensure one is effectively purifying the target(s) of interest (Rowlands et al. 1971).

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